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Attenuation of Cortical Neuronal Apoptosis by Gangliosides[1]

BO RUM RYU, DENNIS W. CHOI,[2] DEAN M. HARTLEY,[3] ERMINIO COSTA,[4] ILO JOU, and BYOUNG JOO GWAG
Department of Pharmacology, Ajou University School of Medicine, Suwon, Kyungkido, Korea (B.R.R., I.J., B.J.G.)

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ABSTRACT

Addition of the natural gangliosides monosialoganglioside (GM1), disialoganglioside, trisialoganglioside, or tetrasialoganglioside in the range of 10 to 100 μM, but not asialoganglioside lacking the sialic acid moiety, attenuated cortical neuronal apoptosis induced by serum deprivation, ionomycin, or cyclosporin A but not by protein kinase inhibitors (staurosporine, genistein, lavendustin A, or herbimycin A). Coaddition of 100 nM wortmannin, a selective inhibitor of phosphatidylinositol 3-kinase, but not 1 μM Go6976, a selective protein kinase C inhibitor, blocked the neuroprotective effect of GM1. In contrast to its antiapoptotic effect, GM1 at up to 200 μM did not attenuate cortical neuronal necrosis induced by exposure to the excitotoxins *N*-methyl-D-aspartate or kainate. Furthermore, GM1 increased the necrosis induced by oxidative stress (addition of Fe^{2+} or buthionine sulfoximine). These data suggest that neuroprotective effects of natural gangliosides may preferentially reflect reduction of neuronal apoptosis rather than necrosis, and be mediated through mechanisms involving activation of phosphatidylinositol 3-kinase.

There has been interest in the ability of the natural gangliosides monosialoganglioside (GM1), disialogangliosides (GD1a, GD1b), and trisialoganglioside (GT1b) to protect central neurons from death induced by a variety of insults. GM1 attenuates neuronal degeneration induced by hippocampal or cortical aspiration (Sofroniew et al., 1986; Sabel et al., 1988), exposure to 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (Hadjiconstantinou and Neff, 1988; Schneider et al., 1992), or ischemia (Seren et al., 1990). In vitro, GM1 or GT1b attenuates excitotoxic neuronal death (Vaccarino et al., 1987; Dawson et al., 1995), and enhances the survival of sympathetic neurons and PC12 cells deprived of nerve growth factor (Ferrari et al., 1993). Several mechanisms have been suggested to underlie the neuroprotective effects of gangliosides, including inhibition of protein kinase C (PKC) translocation (Vaccarino et al., 1987), inhibition of nitric oxide synthase (Dawson et al., 1995), and phosphorylation of the nerve growth factor receptor Trk (Ferrari et al., 1995; Rabin and Mocchetti, 1995).

Previous studies of the neuroprotective effects of natural gangliosides largely precede field attention to the nature of neuronal death under study, necrosis versus apoptosis. Apoptosis was originally recognized by the marked condensation of cytoplasm and nucleus during physiological and pathological processes (Kerr et al., 1972). Internucleosomal DNA fragmentation (DNA ladders) and death depending on protein synthesis have been coined as functional features of apoptosis (Wyllie et al., 1984). Target or growth factor deprivation is highly likely to cause neuronal apoptosis. However ischemia, excitotoxicity, or oxidative stress may induce either necrosis or apoptosis, depending on the system used, and variables such as developmental age or insult intensity (Johnson et al., 1996; Gwag et al., 1999).

This distinction between necrosis versus apoptosis might be important to make, as these different deaths exhibit different responses to pharmacological intervention. For example, neurotrophins attenuate neuronal apoptosis, but potentiate several forms of necrosis arising from oxygen-glucose deprivation, *N*-methyl-D-aspartate (NMDA) treatment, or oxidative stress (Gwag et al., 1995; Koh et al., 1995a; Park et al., 1998).

We set out to test whether gangliosides would reduce cortical neuronal necrosis and apoptosis equally, using a culture system where we have previously established paradigms for inducing each type of death: 1) serum deprivation, ionomycin, or cyclosporin A, inducing neuronal apoptosis associated with cell body shrinkage, aggregation, and condensation of

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[2] Current address: Department of Neurology and Center for the Study of Nervous System Injury, Box 8111, Washington University School of Medicine, St. Louis, MO 63110.
[3] Current address: Center of Neurologic Diseases, Brigham and Women's Hospital, Harvard Institutes of Medicine, Boston, MA 02115.
[4] Current address: Psychiatric Institute, University of Illinois Medical School, Chicago, IL 60612.

ABBREVIATIONS: GM1, monosialoganglioside; BDNF, brain-derived neurotrophic factor; BSO, buthionine sulfoximine; GD1a, disialoganglioside 1a; GQ1b, tetrasialoganglioside 1b; GT1b, trisialoganglioside 1b; NMDA, *N*-methyl-D-aspartate; PI3-K, phosphatidylinositol 3-kinase; PKC, protein kinase C; MEM, minimum essential medium; DIV, days in vitro; LDH, lactate dehydrogenase.

nuclear chromatin, and prevented by inhibitors of protein synthesis or caspase; and 2) exposure to excitotoxins or oxidative stress, inducing neuronal necrosis associated with early swelling of cell body and mitochondria, and insensitivity to inhibitors of protein synthesis or caspase (Gottron et al., 1997; Gwag et al., 1997). An abstract has appeared (Ryu et al., 1996).

Materials and Methods

Cell Cultures

Mouse cortical cell cultures were prepared as described previously (Choi, 1987). Neocortices of 14- or 15-day-old fetal mice were dissociated, plated on a poly-D-lysine- and laminin-coated 24-well plate at a density of four hemispheres per plate (approximately 5×10^4 cells in each well), in minimum essential medium (MEM, Earle's salts) supplemented with 5% horse serum, 5% fetal bovine serum, 21 mM glucose, and 2 mM glutamine. The plates were then maintained up to 7 to 15 days in a humidified incubator with 5% CO_2 at 37°C. For cocultures of neurons and glia, proliferation of non-neuronal cells was halted by exposing cultures to 10 μM cytosine arabinofuranoside at 7 to 9 days in vitro (DIV 7–9) for 2 to 3 days. Cultures were then shifted into a growth medium identical with the plating media, but lacking fetal bovine serum. For neuron-rich cortical cultures, cytosine arabinofuranoside (final concentration, 2.5 μM) was added to cultures at DIV 3.

Assay of trk Phosphorylation

The trk phosphorylation assay was performed as described previously except for minor modifications for the cell culture experiment (Holtzman et al., 1996). Neuron-rich cell cultures (DIV 7) were exposed to 100 ng/ml brain-derived neurotrophic factor (BDNF) or 50 μM GM1 for the indicated points of time. Cultures were then lysed in lysis buffer containing 50 mM Tris, pH 7.5, 1% NP-40, 0.1% SDS, 0.5% deoxycholic acid, 150 mM NaCl, 2 mM phenylmethylsulfonyl fluoride, 100 μg/ml leupeptin, and 0.5 mM Na_2VO_3. Protein concentration was determined using the protein assay kit (Dc protein assay, Bio-Rad Laboratories, Inc., Richmond, CA) and 600 μg protein extract for each condition was immunoprecipitated with 5 μg pan-Trk antibody (Santa Cruz Biotechnologies, Santa Cruz, CA). Precipitates were collected with protein A-sepharose, washed in lysis buffer, subjected to electrophoresis on a 6% SDS-polyacrylamide gel and transferred to a nitrocellulose membrane. The trk tyrosine phosphorylation was detected using 4G10, a phosphotyrosine antibody (UBI, Lake Placid, NY), and an enhanced chemiluminescence system (ECL, Amersham, UK).

Induction of Neuronal Death

Apoptosis. In neuron-rich (>95%) cortical cultures (DIV 7), serum deprivation produces widespread neuronal apoptosis (Gwag et al., 1997). In brief, neuron-rich cortical cultures (DIV 7) were deprived of serum for 24 h by placing cultures in MEM supplemented with 21 mM glucose and 1 μM MK-801, a selective NMDA antagonist. MK-801 was included in all experimental conditions including the serum control to prevent the NMDA neurotoxicity that often occurs with serum, presumably due to glutamate in the serum.

In cocultures of neurons and glia (DIV 10–12) where neurons survive well against serum deprivation, neuronal apoptosis was induced by continuous exposure to 250 nM ionomycin, a selective calcium ionophore, or 20 μM cyclosporin A, a specific inhibitor of serine/threonine protein phosphatase 2B as reported previously (McDonald et al., 1996; Gwag et al., 1999), 100 nM staurosporine, 100 μM genistein, 10 μM lavendustin A, or 3 μM herbimycin A in MEM supplemented with 21 mM glucose. Treatment with each of these protein kinase inhibitors was shown to produce apoptosis of cortical neurons (Koh et al., 1995b; Behrens et al., 1995).

Excitotoxicity. For fast excitotoxicity, cocultures of neurons and glia (DIV 12–14) were exposed to 300 μM NMDA for 10 min in HEPES controlled salt solution: 120 mM NaCl, 54 mM KCl, 0.8 mM $MgCl_2$, 1.8 mM $CaCl_2$, 15 mM glucose, 20 mM HEPES, 10 mM NaOH, and 0.5% phenol red, as reported previously (Koh et al., 1995a). To trigger relatively slow excitotoxicity, cocultures of neurons and glia (DIV 12–14) were exposed to 15 μM NMDA or 40 μM kainate for 24 h in MEM supplemented with 21 mM glucose (Gwag et al., 1997).

Oxidative Stress. Cocultures of neurons and glia (DIV 12–14) were exposed to 30 μM $FeCl_2$ or 1 mM buthionine sulfoximine (BSO) for 24 h in MEM supplemented with 21 mM glucose. This treatment produces mild oxidative neuronal necrosis over 24 h (Gwag et al., 1995).

Analysis of Neuronal Death

For DNA staining, cultures were incubated in 0.5 μg/ml propidium iodide and examined under rhodamine filter. Neuronal death was analyzed by measuring efflux of lactate dehydrogenase (LDH) into the bathing medium 24 h later as reported previously, with the exception of serum deprivation-induced apoptosis where LDH assay was avoided due to high levels of LDH in serum (Koh et al., 1995a). Neuronal loss after serum deprivation was analyzed 24 h later by counting viable neurons with normal cell body and processes after staining with hematoxylin-eosin Y, which correlates well with the trypan blue exclusion method.

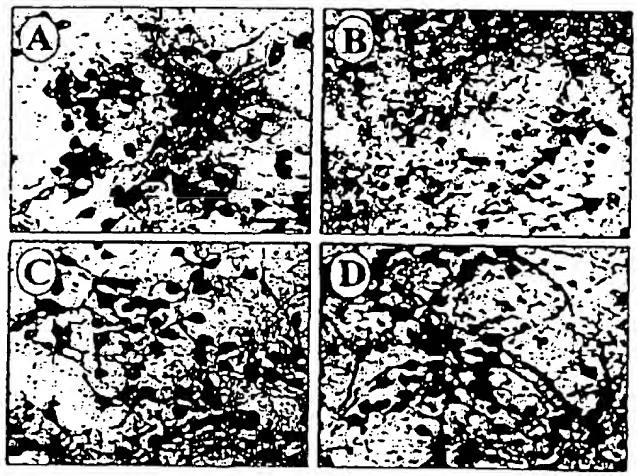
Reagents

All gangliosides were donated by Fidia (Italy). NMDA and MK-801 were purchased from Research Biochemicals, Inc. (Natick, MA). Wortmannin, genistein, lavendustin A, herbimycin A, and Go6976 were purchased from Calbiochem (San Diego, CA). Kainate, BSO, $FeCl_2$, cycloheximide, cytosine arabinofuranoside, cyclosporin A, and staurosporine were purchased from Sigma (St. Louis, MO). BDNF was purchased from Peprotec (London, UK).

Results

Gangliosides Attenuate Neuronal Apoptosis: Dependence on Activity of Tyrosine Kinase. Neuron-rich cortical cultures (DIV 7–8) deprived of serum underwent apoptosis characterized by cell body shrinkage, chromatin condensation, and cycloheximide sensitivity (Gwag et al., 1997; Fig. 1, A–D). Continuous inclusion of 10 to 100 μM GM1 attenuated serum deprivation-induced neuronal cell apoptosis in a dose-dependent manner (Fig. 1E). Inclusion of 10 to 100 μM GD1a, 10 μM GT1b, or 10 μM tetrasialoganglioside (GQ1b), but not 10 μM asialoganglioside, also protected cortical neurons against serum deprivation-induced apoptosis (Table 1). Treatment with 50 μM GM1 additionally attenuated neuronal apoptosis induced by 24-h exposure of DIV 10–12 cultures to 20 μM cyclosporin A or 250 nM ionomycin (Fig. 2E-I) that was blocked by addition of 1 μg/ml cycloheximide as previously reported (Mcdonald et al. 1996; Gwag et al., 1999).

In contrast with its protective effects against several forms of apoptosis, 50 μM GM1 did not protect neurons in cocultures of neurons and glia (DIV 10–12) from undergoing apoptosis induced by 24-h exposure to 100 nM staurosporine (Koh et al., 1995b), or the tyrosine kinase inhibitors (Behrens et al., 1995), 100 μM genistein, 10 μM lavendustin A, or 3 μM herbimycin A (Fig. 2I). All of these deaths were also characterized by early cell body shrinkage, chromatin condensation, and sensitivity to cycloheximide (Fig. 2, A–D and I).



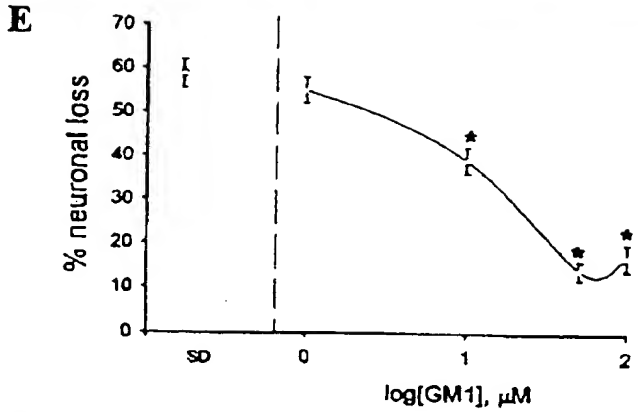


Fig. 1. Natural gangliosides attenuate apoptosis of cortical neurons deprived of serum. Bright field photomicrographs of hematoxylin-eosin-stained cortical neurons (DIV 8) maintained for 24 h in serum-containing media (A), serum-free media (B), serum-free media containing 1 μg/ml cycloheximide (C), or serum-free media containing 50 μM GM1 (D). Arrows point to shrunken, degenerating neurons. Bar, 50 μm. E, sister cultures were deprived of serum, alone serum deprivation or in the presence of indicated concentrations of GM1. Neuronal death was assessed by counting viable neurons 24 h later after the onset of serum deprivation, using hematoxylin-eosin staining. mean ± S.E.M. (n = 16 fields randomly chosen in four culture wells per condition), in comparison with the number of neurons incubated in serum-containing medium (0% neuronal death). * indicates significant difference from serum-free condition at $P < .05$, using ANOVA and Student-Newman-Keuls' test.

Neuroprotective Action of GM1 Differs from That of Neurotrophins. The possibility that ganglioside blockade of serum deprivation-induced apoptosis was mediated by signaling pathways common to those activated by neurotrophins was considered. As reported previously (Ghosh et al., 1994; Koh et al., 1995a), addition of 100 ng/ml BDNF protected cortical neurons from apoptosis induced by serum deprivation. This exposure to BDNF was associated with rapid phosphorylation of Trk B within 15 min (Fig. 3). However, Trk phosphorylation (as revealed by a pan Trk antibody) was not observed over 3 h after exposure to 50 μM GM1 (Fig. 3). Furthermore, the neuroprotective effect of BDNF, but not GM1, was blocked by inclusion of 1 μM Go 6976, an agent known to inhibit PKC α, β1, and γ (Martiny-Baron et al., 1993; Fig. 4). Addition of 100 nM wortmannin, a selective inhibitor of phosphatidylinositol 3-kinase (PI3-K; Arcaro and Wymann, 1993) lacked intrinsic neurotoxicity but reversed the neuroprotective effect of GM1 while not affecting that of BDNF (Fig. 4).

TABLE 1

Neuroprotective effect of gangliosides against serum deprivation-induced neuronal death

Neuron-rich cortical cultures (DIV 7) were deprived of serum, alone serum deprivation, or in the presence of 1 μg/ml cycloheximide (CHX). Sister cultures were exposed to serum deprivation in the presence of the indicated concentrations of GD1a, GT1b, GQ1b, or asialoganglioside. Viable neurons were counted 24 h later after staining with hematoxylin-eosin, mean ± S.E.M. (n = 16 fields randomly chosen in four culture wells per condition), scaled to the mean number of surviving cells incubated in serum-containing medium (0% neuronal death).

Conditions	Neuronal Death
	%
Serum deprivation	69 ± 3
+ CHX	13 ± 5*
+ GD1a	
10 μM	38 ± 3*
100 μM	39 ± 4*
+ GT1b, 10 μM	38 ± 3*
+ GQ1b, 10 μM	31 ± 4*
+ aGM1, 10 μM	64 ± 4

*Mean ± S.E. indicates significant difference from serum deprivation at $P < .05$, using ANOVA and Student-Newman-Keuls' test.

GM1 Does Not Attenuate Excitotoxic Neuronal Necrosis in Cortical Cell Cultures. Cortical neurons in our cultures exposed to NMDA or kainate die predominantly via necrosis, characterized by prominent early cell body swelling and insensitivity to antiapoptosis agents such as cycloheximide, caspase inhibitors, and growth factors (Gwag et al., 1997; Gottron et al., 1997). A 2-h pretreatment or cotreatment with 50 to 200 μM GM1 did not influence the rapidly triggered excitotoxic necrosis induced by 10-min exposure to 300 μM NMDA, or the slowly triggered excitotoxic necrosis induced by 24-h exposure to 15 μM NMDA or 40 μM kainate (Table 2).

GM1 Potentiates Oxidative Neuronal Necrosis. Cortical or striatal cell cultures exposed to 30 μM Fe^{2+} developed widespread neuronal necrosis characterized by prominent cell body swelling and insensitivity to cycloheximide (Gwag et al., 1995; Park et al., 1998). The Fe^{2+}-induced neuronal necrosis was not attenuated by 24-h pretreatment or cotreatment with 10 to 100 μM GM1 (data not shown). Rather, cotreatment with 50 μM GM1 potentiated submaximally induced oxidative neuronal death 24-h after exposure to 30 μM Fe^{2+} or 1 mM BSO (Table 2). Cotreatment with 10 or 50 μM GT1b also potentiated Fe^{2+}-induced neuronal death (data not shown).

Discussion

The major finding of the present study is that the protective effect of gangliosides on cortical neurons was selective for certain forms of apoptosis, induced by serum deprivation, ionomycin, or cyclosporin A, and did not extend to the neuronal necrosis induced by excitotoxic (Csernansky et al., 1994; Gwag et al., 1997) or oxidative (Gwag et al., 1995; Park et al., 1998) insults. Rather, GM1 exposure enhanced oxidative neuronal death, a surprising observation in view of the known direct antioxidant activity of gangliosides (Tyurin et al., 1992; Maulik et al., 1993).

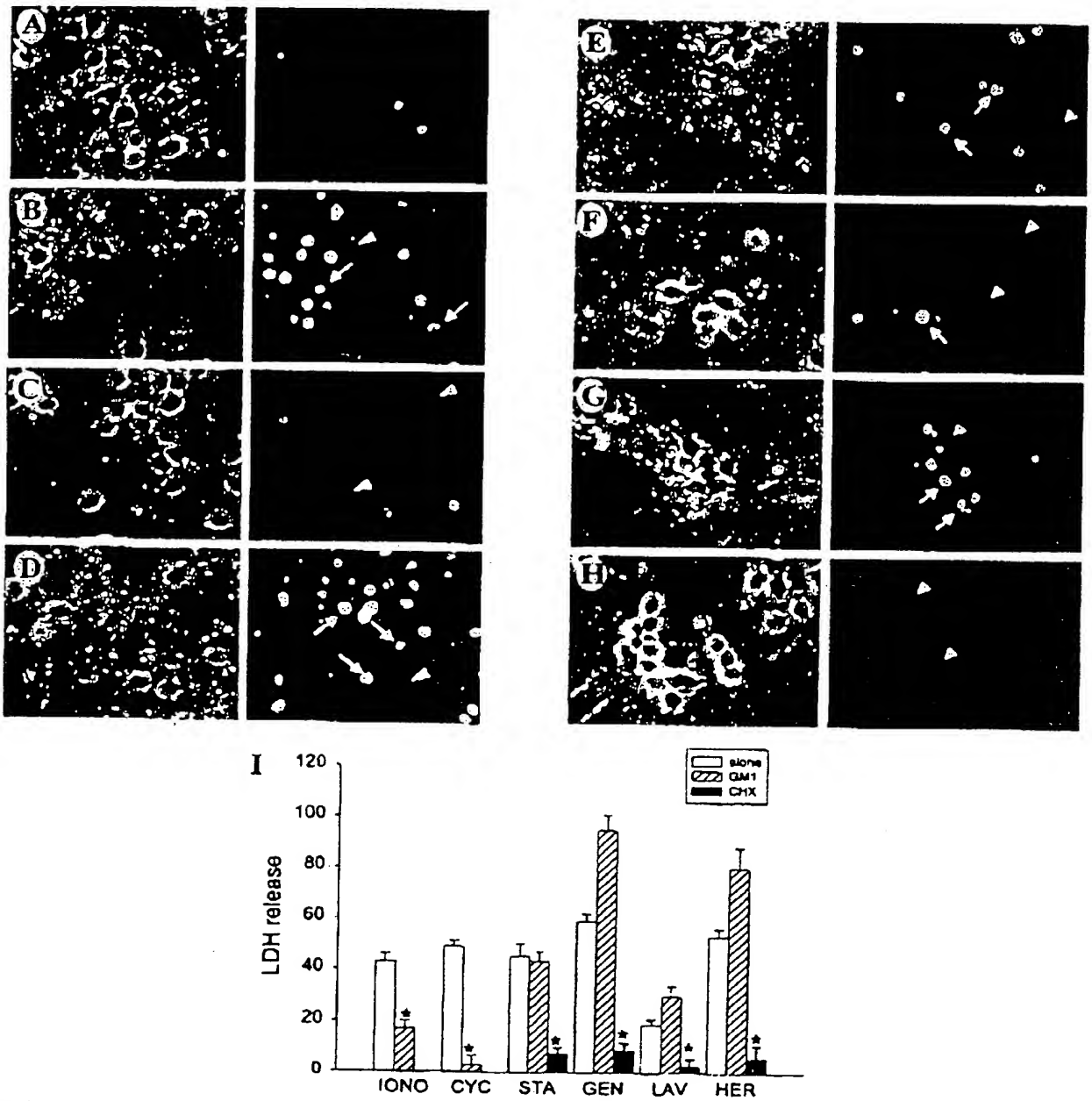


Fig. 2. GM1 does not attenuate neuronal apoptosis induced by staurosporine or tyrosine kinase inhibitors. Phase contrast (left) and fluorescence (right; propidium iodide staining) photomicrographs of mixed cortical cell cultures (DIV 11) taken 24 h after sham wash (A), or exposure to 100 μM genistein, alone (B) or in the presence of 1 μg/ml cycloheximide (C) or 50 μM GM1 (D). Sister cultures were exposed to 20 μM cyclosporin A (E), cyclosporin A plus 50 μM GM1 (F), 250 nM ionomycin (G), or ionomycin plus 50 μM GM1 (H). Compared with normal neurons (arrow heads), apoptotic neurons were marked by cell body shrinkage and chromatin condensation (arrows). Bar denotes 50 μm. I, cortical cell cultures (DIV 10–12) were exposed to 250 nM ionomycin (IONO), 20 μM cyclosporin A (CYC), 100 nM staurosporine (STA), 100 μM genistein (GEN), 10 μM lavenduatin A (LAV), or 3 μM herbimycin A (HER), alone or in the presence of 1 μg/ml cycloheximide (CHX) or 50 μM GM1 (GM1). Neuronal injury was analyzed 24 to 28 h later by measuring LDH efflux in the bathing media, mean ± S.E.M. (n = 8–12 cultures per condition), scaled to the mean LDH value corresponding to the near complete neuronal death induced by exposure to 500 μM NMDA for 24 h (= 100%). • indicates significant decline from alone at $P < .05$, using ANOVA and Student-Newman-Keuls' test.

Furthermore, the antiapoptosis effects of GM1 did not extend to apoptosis induced by several tyrosine kinase inhibitors. Additional study will be necessary to determine whether ganglioside neuroprotection is truly specific for certain forms of programmed cell death, or whether, probably more likely, gangliosides failed to block apoptosis induced by tyrosine kinase inhibitors because these inhibitors interfere with the transduction of ganglioside signaling.

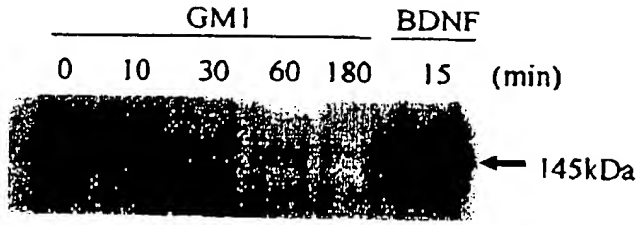


Fig. 3. Lack of Trk phosphorylation after the addition of GM1 gangliosides in cortical cell cultures. An immunoblot labeled with 4G10 against phosphotyrosine. Treatment with 50 μM GM1 for 10 to 180 min did not induce tyrosine phosphorylation of the Trks in neuron-rich cell cultures (DIV 7), whereas addition of 100 ng/ml BDNF did induce Trk phosphorylation 15 min later.

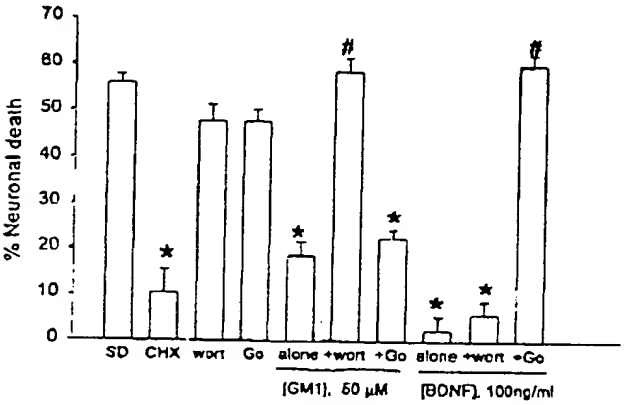


Fig. 4. Involvement of PI3-K for the antiapoptosis action of GM1. Neuron-rich cortical cultures (DIV 7) were deprived of serum, alone (serum deprivation) or in the presence of 1 μg/ml cycloheximide (CHX), 1 μM Co 6976 (Go) or 100 nM wortmannin (wort). During serum deprivation, sister cultures were added with 50 μM GM1 or 100 ng/ml BDNF, alone or in the presence of 1 μM Go 6976 (+Go) or 100 nM wortmannin (+wort). Neuronal death was analyzed 24 h later as described in the legend to Fig. 1. mean ± S.E.M (n = 4 culture wells for condition). • significant reduction from serum deprivation, #significant difference from GM1 alone or BDNF alone, at $P < .05$, using ANOVA and Student-Newman-Keuls' test.

The transduction of the antiapoptotic effect of GM1 appeared to be at least partly different from that of BDNF. GM1 neuroprotection, unlike BDNF neuroprotection, was not associated with trk phosphorylation, not blocked by the PKC inhibitor Go 6976 (Gwag and Choi, 1996), and was blocked by nontoxic exposure to the putative selective PI3-K inhibitor, wortmannin. This conclusion is at variance with the suggestion that the neurotrophic effects of gangliosides are mediated through activation of Trk receptors (Ferrari et al., 1995; Rabin and Mocchetti, 1996), but fits with recent suggestions that these effects may be mediated through activation of the extracellular signal-regulated kinase subgroup of mitogen-activated protein kinase, p70 S6 kinase, or Src family tyrosine kinase Lyn (Kasahara et al., 1997; Van Brocklyn et al., 1997).

The failure of GM1 to attenuate NMDA- or kainate- induced excitotoxic neuronal death in the present experiments contrasts with other studies that have observed antiexcitotoxic effects of gangliosides in cerebellar and cortical cell cultures (Vaccarino et al., 1987; Dawson et al., 1995). Although further experiments will be needed to establish the basis for these differences, as a working hypothesis we propose that programmed cell death may have played a larger

TABLE 2

Effect of GM1 on neuronal necrosis triggered by excitotoxins or oxidative stress

Cocultures of neurons and glia (DIV 12–14) were exposed to 300 μM NMDA for 10 min, alone or after a 2-h pretreatment with indicated concentrations of GM1. Sister cultures were exposed to 15 μM NMDA, 40 μM kainate, 30 μM Fe^{2+}, or 1 mM BSO for 24 h, alone or in the presence of 50 μM GM1. Neuronal death was analyzed 24 h later by measuring LDH efflux in the bathing media, mean ± S.E. (n = 8 to 12 cultures per condition), scaled to the mean LDH value corresponding to near-complete neuronal death induced after continuous exposure to 600 μM NMDA for 24 h (= 100%).

Conditions	Neuronal Death
	%
NMDA, 300 μM	
Alone	49 ± 7
+ GM1	
10 μM	37 ± 7
50 μM	48 ± 10
200 μM	48 ± 5
NMDA, 15 μM	
Alone	23 ± 3
+ GM1, 50 μM	24 ± 6
Kainate, 40 μM	
Alone	48 ± 3
+ GM1, 50 μM	60 ± 3
Fe^{2+}, 30 μM	
Alone	19 ± 9
+ GM1, 50 μM	49 ± 8*
BSO, 1 mM	
Alone	7 ± 1
+ GM1, 50 μM	30 ± 3*

*Mean ± S.E. indicates significant difference from relevant control (alone) at $P <$ 05, using ANOVA and Student-Newman-Keuls' test.

role in excitotoxicity in the other systems, compared with our system, and that the beneficial effect of gangliosides may primarily reflect attenuation of this programmed cell death. Several authors have emphasized that neuronal apoptosis can occur after glutamate receptor overactivation (Ankarcrona et al., 1995; Portera-Cailliau et al., 1996). As noted above, factors such as developmental age and insult intensity influence the extent to which programmed cell death contributes to excitotoxicity, with immaturity and lower insult levels (and perhaps in particular the nonreceptor-mediated form of glutamate toxicity reflecting reduced cysteine uptake; Miyamoto et al., 1989), favoring this contribution. In support of this, treatment with glutamate can induce apoptosis in very young (DIV 3–4) cultured cortical neurons (Kure et al., 1991), but predominantly necrosis in older cultured cortical neurons (Gwag et al., 1997; Sohn et al., 1998).

Although gangliosides have been proposed as neuroprotective agents capable of reducing brain or spinal cord damage in several disease conditions, the present study raises the possibility that these drugs may be of neuroprotective value only in conditions where apoptosis is prominent, and raises the caution that gangliosides may even be deleterious in conditions where free radical-mediated necrosis is prominent.

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Send reprint requests to: Dr. Byoung Joo Gwag, Department of Pharmacology, Ajou University School of Medicine, Suwon, Kyungkido, Korea 442-749. E-mail: bjgwag@madang.ajou.ac.kr